

Factors triggering type III secretion in *Pseudomonas aeruginosa*

Jaewha Kim,^{1†‡} Kyungseop Ahn,^{1†‡} Sungran Min,¹ Jinghua Jia,¹ Unhwan Ha,¹ Donghai Wu² and Shouguang Jin¹

¹Department of Molecular Genetics and Microbiology, PO Box 100266, University of Florida, Gainesville, FL 32610, USA

²Guangzhou Institute of Biomedicine and Health, Chinese Academy of Sciences, Guangzhou, Guangzhou, China

The type III secretion system of *Pseudomonas aeruginosa* is tightly regulated by various environmental signals, such as low calcium and contact with the host cell. However, the exact signals triggering type III secretion are unknown. The present study describes the finding that secretion of *P. aeruginosa* type III effector molecules requires protein factors from serum and L broth, designated type III secretion factors (TSFs), in addition to the low-calcium environment. In the absence of TSF or calcium chelator EGTA, basal levels of type III effector molecules are accumulated intracellularly. Addition of TSF and EGTA together effectively triggers the secretion of pre-existing effector molecules in a short time, even before the active expression of type III genes; thus, active type III gene expression does not seem to be a prerequisite for type III secretion. A search for TSF molecules in serum and L broth resulted in the identification of albumin and casein as the functional TSF molecules. Although there is no clear sequence similarity between albumin and casein, both proteins are known to have a low-affinity, high-capacity calcium-binding property. Tests of well-studied calcium-binding proteins seemed to indicate that low-affinity calcium-binding proteins have TSF activity, although the requirement of low-affinity calcium-binding ability for the TSF activity is not clear. *P. aeruginosa* seems to have evolved a sensing mechanism to detect target cells for type III injection through host-derived proteins in combination with a low-calcium signal. Disruption of the bacterial ability to sense low calcium or TSF might be a valid avenue to the effective control of this bacterial pathogen.

INTRODUCTION

The remarkable ability of *Pseudomonas aeruginosa* to adapt to and thrive in a wide variety of environments contributes significantly to the ability of this bacterium to cause various human infections (Bodey *et al.*, 1983; Holder, 1993; Pier, 2002). Unlike most human pathogens possessing highly restricted host ranges, *P. aeruginosa* is pathogenic not only to humans, but also to *Caenorhabditis elegans*, *Drosophila* and *Arabidopsis thaliana* (Rahme *et al.*, 1995; D'Argenio *et al.*, 2001; Aballay & Ausubel, 2002). This broad host range is contributed in part by the numerous virulence factors

encoded by *P. aeruginosa*, including exotoxin A, phospholipase C, alkaline protease, elastase, alginate, pyocyanin, pili and non-pilus adhesins (Ramphal *et al.*, 1991; Deretic *et al.*, 1995; Lory & Strom, 1997; Vasil & Ochsner, 1999). *P. aeruginosa*, like many other Gram-negative animal and plant pathogens, also encodes a type III secretion machinery, where over 30 proteins assemble into a complex designed to deliver effector molecules directly into the cytoplasmic compartment of eukaryotic cells (Yahr *et al.*, 1995; Frank, 1997). Injection of the bacterial effector molecules into the host cells results in various physiological changes, all of which seem to confer a survival advantage on the bacterial pathogen within the host environment (Hueck, 1998; Muller *et al.*, 2001). In animal pathogens, the principal function of the type III secretion system (TTSS) is to deliver anti-host virulence determinants into mammalian cells, thus not only evading the host's immune system, but also acquiring essential nutrients. Similarly, plant pathogens utilize TTSS to cause disease in susceptible plants and to trigger a hypersensitive response in resistant plants (Hueck, 1998; Muller *et al.*, 2001).

†These authors contributed equally to this work.

‡Present address: Korea Research Institute of Bioscience and Biotechnology, Taejeon 305-600, Republic of Korea.

Abbreviations: Ap, ampicillin; Cb, carbenicillin; Gm, gentamicin; HRP, horseradish peroxidase; HSA, human serum albumin; NTA, nitrilotriacetic acid; OPD, o-phenylenediamine; Sm, streptomycin; Sp, spectinomycin; TCA, trichloroacetic acid; TSF, type III secretion factor; TTSS, type III secretion system.

TTSS of *P. aeruginosa* responds to various environmental signals, such as low calcium and direct contact with tissue-culture cells (Yahr *et al.*, 1995; Frank, 1997). Upon activation, the type III secretion apparatus translocates effector molecules into the cytoplasm of host cells, resulting in cell rounding and lifting and cell death by necrosis or apoptosis (Finck-Barbancon *et al.*, 1997; Pederson *et al.*, 1999; Kaufman *et al.*, 2000). There are four known effector molecules, including ExoS and ExoT, two homologous toxins with both ADP-ribosyltransferase and GTPase-activating protein activities, an acute cytotoxin, ExoU, with lipase activity, and an adenylate cyclase, ExoY (Yahr *et al.*, 1996, 1998; Finck-Barbancon *et al.*, 1997; Hauser *et al.*, 1998; Sato *et al.*, 2005). It is well known that ExoS preferentially ADP-ribosylates several Ras family members (GTP-binding proteins) required for the regulation of intracellular vesicle transport, cell proliferation and differentiation (Coburn & Gill, 1991; Ganesan *et al.*, 1998). The ADP-ribosyltransferase activity of ExoS has also been shown to be essential in triggering programmed cell death in various types of tissue-culture cells (Kaufman *et al.*, 2000; Jia *et al.*, 2003).

The type III secretion complex of *P. aeruginosa* is highly similar to that of members of the genus *Yersinia* and, when expressed in cells of this genus, the ExoS protein can be translocated into mammalian cells via the yersinia TTSS (Frithz-Lindsten *et al.*, 1997, 1998). Both organisms harbour multiple regulators to tightly control the expression of the large type III secretion gene clusters in response to low-calcium environmental stimuli. The mechanism by which the extracellular calcium concentration is translated into a transcriptional signal remains a mystery. In *P. aeruginosa*, the expression of type III-related genes is regulated

coordinately by a transcriptional activator, ExsA (Yahr & Frank, 1994; Hovey & Frank, 1995). ExsA is a DNA-binding protein that recognizes a consensus sequence (TNAANA) located approximately 51–52 bp upstream of the transcriptional start site to stimulate the expression of type III genes, including the *exsA* gene itself. More recently, a number of genes have been shown to affect the expression of type III genes, including adenylcyclase (*cyaA*), pseudouridine (*truA*) and pyruvate dehydrogenase (*aceAB*) (Dacheux *et al.*, 2002; Wolfgang *et al.*, 2003; Ahn *et al.*, 2004). Meanwhile, the type III genes are also regulated negatively by ExsD, the RhlI/RhlR quorum-sensing system and stationary-phase sigma factor RpoS (McCaw *et al.*, 2002; Hogardt *et al.*, 2004).

In this report, we describe the finding that optimal secretion of *P. aeruginosa* type III effector molecules requires a protein factor, designated type III secretion factor (TSF), in addition to the calcium chelator. The TSFs in L broth and serum were identified as caseins and albumin, respectively. Further analysis revealed that low-affinity calcium-binding proteins have TSF activity, whilst high-affinity calcium-binding proteins do not. Significance of the current finding in understanding the pathogen–host interaction is discussed.

METHODS

Bacterial strains and plasmids. Bacterial strains and plasmids used in this study are listed in Table 1. *P. aeruginosa* was grown in Luria (L) agar or L broth at 37 °C. Antibiotics were used at final concentrations of 150 µg carbenicillin (Cb) ml⁻¹, 150 µg gentamicin (Gm) ml⁻¹, 200 µg streptomycin (Sm) ml⁻¹ and 200 µg spectinomycin (Sp) ml⁻¹ for *P. aeruginosa*, and 100 µg ampicillin (Ap) ml⁻¹,

Table 1. Strains and plasmids used in this study

Ap^r, Ampicillin-resistance marker; Gm^r, gentamicin-resistance marker; Sp^r, spectinomycin-resistance marker; Sm^r, streptomycin-resistance marker; Tc^r, tetracycline-resistance marker.

Strain/plasmid	Description	References or source
<i>P. aeruginosa</i> strains		
PAK	Clinical isolate of wild-type invasive strain	David Bradley (Memorial University of Newfoundland, Canada)
PAK <i>exoT</i> ::Gm	PAK with chromosomal disruption of the <i>exoT</i> locus; Gm ^r	Kaufman <i>et al.</i> (2000)
PAK <i>exoS</i> ::Ω <i>exoT</i> ::Gm	PAK with chromosomal disruption of the <i>exoS</i> and <i>exoT</i> loci; Sp ^r Sm ^r Gm ^r	Kaufman <i>et al.</i> (2000)
PAK <i>exsA</i> ::Ω	PAK with chromosomal disruption of the <i>exsA</i> locus with Ω cassette; Sp ^r Sm ^r	Frank <i>et al.</i> (1994)
Plasmids		
pDN19 <i>lac</i> Ω	Promoterless <i>lacZ</i> -fusion vector; Sp ^r Sm ^r Tc ^r	Totten & Lory (1990)
pHW0005	PAK <i>exoS</i> promoter fused to <i>lacZ</i> in pDN19 <i>lac</i> Ω; Sp ^r Sm ^r Tc ^r	Ha & Jin (2001)
pHW0006	PAK <i>exoT</i> promoter fused to <i>lacZ</i> in pDN19 <i>lac</i> Ω; Sp ^r Sm ^r Tc ^r	Ha & Jin (2001)
pHW0032	PAK <i>exsA</i> promoter fused to <i>lacZ</i> in pDN19 <i>lac</i> Ω; Sp ^r Sm ^r Tc ^r	Ha <i>et al.</i> (2004)
pUCP19	Broad-host-range shuttle vector; Ap ^r	Schweizer (1991)
pHW0029	ExoS-FLAG of PAK in pUCP19; Ap ^r	Ha & Jin (2001)

25 µg Sm ml⁻¹ and 50 µg Sp ml⁻¹ for *Escherichia coli*. All of the calcium-binding proteins used in this study were purchased from Sigma.

Activation of TTSS and detection of ExoS-FLAG by Western blot. In 24-well plates, each well was filled with 360 µl Dulbecco's modified Eagle's medium (DMEM) plus 5 mM EGTA and 1% fetal calf serum (FCS) or 10% L broth containing 10⁸ bacterial cells from fresh overnight cultures. After 3 h incubation at 37 °C in a CO₂ incubator, ExoS-FLAG secreted into the culture supernatant or intracellular ExoS-FLAG was detected by Western blot analysis. Secreted proteins in the culture supernatant were precipitated by the addition of trichloroacetic acid (TCA) to 15% and kept on ice for 30 min. The precipitated proteins from 250 µl supernatant were collected by centrifugation, washed three times with acetone, resuspended in 50 µl sample buffer and subjected to SDS-PAGE. Equal loading was based on samples from the same number of bacterial cells. Protein bands were transferred onto a PVDF membrane by using a Bio-Rad electroblotter and the membrane was blocked with 5% non-fat milk in Tris-buffered saline containing 0.1% Tween 20 (TBS-T) for 2 h, followed by incubation with anti-FLAG M2 mAb (Sigma) for 1.5 h. ExoS-FLAG protein was then bound by horseradish peroxidase (HRP)-conjugated anti-mouse IgG as secondary antibody and detected with ECL reagent (Amersham Biosciences).

Purification and quantification of secreted ExoS-FLAG. Strain PAKexoST/pHW0029 was grown overnight in 100 ml L broth supplemented with 5 mM EGTA. Bacterial cells were collected by centrifugation at 15 000 g for 15 min and the culture supernatant was decanted into a new container with an equal volume of saturated ammonium sulfate and kept at 4 °C overnight. Protein precipitates were recovered by centrifugation and dissolved in 10 ml PBS. The protein sample was mixed with an anti-FLAG antibody and incubated at 4 °C for 2 h, followed by an additional 2 h incubation with 0.1 vol. protein G-agarose. The beads were washed twice with PBS-T buffer (100 mM phosphate buffer, 150 mM NaCl, 0.2% BSA and 0.05% Tween 20) and the bound ExoS-FLAG was eluted by adding 100 µg FLAG peptide ml⁻¹ (Sigma) and reacting for 20 min at room temperature with gentle agitation. The quantity of eluted ExoS-FLAG protein was measured by the bicinchoninic acid (BCA) protein-assay method. As a standard marker, serially diluted BSA solutions were reacted for 30 min at 37 °C with the BCA reagent mixture (Pierce). A₅₆₀ values of the reactions in the plates were measured to calculate the amount of ExoS-FLAG in the samples. The quantified ExoS-FLAG was also evaluated by SDS-PAGE and Western blot analysis.

Sandwich ELISA for detection of ExoS-FLAG in bacterial-culture supernatant. As capturing antibody, rabbit anti-ExoS antibody was diluted in 0.1 M carbonate buffer (pH 9.6) and used to coat 96-well microtitre plates (Nunc). After overnight incubation at 4 °C, the plates were washed three times with PBS-T and each well was blocked with 50 µl 2% BSA (Sigma) in PBS for 2 h at 37 °C. The plates were then washed and 50 µl culture supernatant or positive control (serial dilutions of a known amount of purified ExoS-FLAG) was applied to each well. The plates were incubated at 37 °C for 1 h, washed and then 50 µl mouse anti-FLAG M2 mAb (diluted 2000-fold in PBS-T) was added to each well. After incubation at 37 °C for 1 h and washing, 50 µl HRP-conjugated goat anti-mouse IgG diluted in PBS-T was added to each well. The plates were incubated at 37 °C for 1 h, washed and then 50 µl citrate/phosphate buffer (pH 5.0) containing *o*-phenylenediamine (OPD; Sigma) as peroxidase substrate was added together with H₂O₂. A₄₉₀ was measured with an automated ELISA reader (Dynatech Laboratories) after 30 min incubation at room temperature. Each sample was run in triplicate and the mean value of the A₄₉₀ readings for the triplicate wells was taken. The amount of ExoS-FLAG in each sample was

determined by using a standard curve generated with a known amount of serially diluted ExoS-FLAG protein. ExoS in culture supernatants is shown as ng ml⁻¹ in all figures.

Isolation of type III activating factor. To identify the active fraction of the TSF from serum and L broth, the samples were separated into <10, 10–50, 50–100 and >100 kDa fractions by using corresponding molecular mass cut-off membrane filters (Amicon). A gel-filtration column (Bio-Rad Bio-Silect SEC 250-5, 300 × 7.8 mm) was further used for fractionation of serum and L broth on an HPLC system (Bio-Rad BioLogic DuoFlow system), using 0.1 M sodium phosphate, 0.15 M NaCl, pH 6.8, as the mobile phase. Each fraction was tested for the ability to activate ExoS-FLAG secretion by sandwich ELISA. A 40 µl aliquot of each fraction was added to 360 µl DMEM containing 5 mM EGTA and 10⁸ cells of PAKexoST/pHW0029 in 24-well plates. After incubating for 3 h at 37 °C in a CO₂ incubator, 50 µl aliquots of samples were subjected to sandwich ELISA as described above.

Other methods. A standard β-galactosidase assay (Miller, 1972) was conducted to determine the expression of *exoS::lacZ*, *exsA::lacZ* and *exoT::lacZ* fusion genes. Standard methods were used for plasmid DNA preparation, restriction-enzyme digestion and cloning (Sambrook *et al.*, 1989).

RESULTS

Triggering of type III secretion independently of type III gene expression

Expression of the *P. aeruginosa* type III secretion genes is known to be activated by either a low-calcium growth environment or direct contact with the host cells. To understand the dynamics of type III gene expression and secretion, we tested the expression patterns of the type III secretion genes during overnight culture of *P. aeruginosa* in L broth, which we typically used to infect HeLa cells or subculture into a type III-inducing medium for type III secretion analysis. Plasmids with transcriptional fusions of *exoS*, *exoT* or *exsA* promoter and promoterless *lacZ* were introduced individually into a wild-type *P. aeruginosa* strain, PAK, and β-galactosidase activities were monitored in the resulting strains. As shown in Fig. 1(a–c), expression of *exoS::lacZ* (pHW0005), *exoT::lacZ* (pHW0006) and *exsA::lacZ* (pHW0032) decreased from basal level, then increased gradually after 3 h culture and reached relatively high levels by 15 h even in the absence of EGTA, although much higher levels were reached in the presence of EGTA. Despite the high basal level after 15 h culture without EGTA, no secretion of ExoS or ExoT was observed when the culture supernatants were tested by Western blot analysis (Fig. 2, left column), suggesting that either their intracellular levels did not reach certain required thresholds or that EGTA is essential for secretion. In the presence of EGTA, a high level of ExoS secretion was observed (Fig. 2, left column). The overnight cultures from L broth were washed once with PBS and resuspended in a type III-inducing medium (DMEM + 1% FCS + 5 mM EGTA) with 10-fold dilution. Within the first 3 h incubation at 37 °C, expression of the three reporter genes decreased, indicating no active production of the type III secretion components in the early adaptive-growth phase (Fig. 1d–f). However, within the

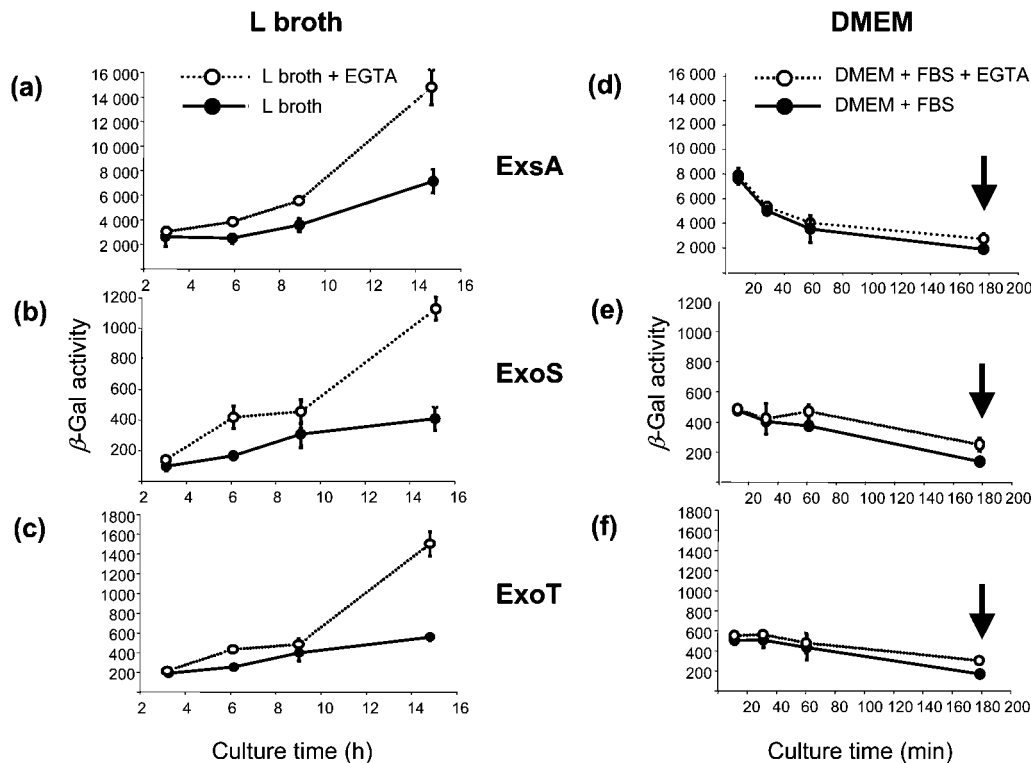


Fig. 1. Expression of type III secretion genes in L broth and a typical type III-inducing medium. To monitor the expression of type III secretion genes, wild-type strain PAK harbouring plasmids with promoter fusions of *exsA::lacZ* (a, d), *exoS::lacZ* (b, e) or *exoT::lacZ* (c, f) were used to measure β -galactosidase activity. Overnight cultures of the bacterial cells grown in L broth were washed in PBS and inoculated into 3 ml fresh L broth or DMEM plus 1% FCS with or without 5 mM EGTA with 10-fold dilution, and grown with or without 5 mM EGTA in a CO₂ incubator at 37 °C without shaking. β -Galactosidase activities were measured at indicated times after incubation. Mean values of triplicate assays are shown.

same time frame, active secretion of ExoS was observed (Fig. 2, right column), suggesting that pre-existing ExoS was secreted in the initial stage. These results further suggested that there is no requirement for a certain threshold level of intracellular ExoS for active secretion; instead, the presence of EGTA seems essential for the secretion, even before active expression of the type III genes. The ExoT secretion pattern was very similar to that of ExoS (data not shown).

Secretion of type III effector ExoS requires both EGTA and factors from serum or L broth

As shown above, secretion of ExoS and ExoT requires EGTA in both L broth and tissue-culture medium (DMEM + 1% FCS); however, almost no ExoS or ExoT secretion was observed when the bacterial cells were grown in minimal medium A (MinA) (Davis & Mingioli, 1950) supplemented with 5 mM EGTA or nitrilotriacetic acid (NTA) (data not shown). To identify the essential components for ExoS secretion present in the tissue-culture medium and L broth, strain PAK*exoT* was grown in various combinations of culture media and Western blot analysis was carried out to detect ExoS in the culture supernatants (for secretion) and bacterial-cell lysates (for its overall expression). Remarkably,

in the presence of calcium chelator EGTA or NTA, ExoS secretion was not detectable in DMEM without FCS, whereas supplementation with serum or L broth resulted in the secretion of a high level of ExoS (Fig. 3). Likewise, addition of serum or L broth to MinA restored ExoS secretion in the presence of the calcium chelators (data not shown), suggesting that serum and L broth contain factors essential for ExoS secretion.

To identify the TSFs present in serum and L broth, a highly sensitive sandwich ELISA system was developed to accurately quantify the secreted ExoS. The sandwich ELISA involves: (i) coating a 96-well plate with a polyclonal rabbit anti-ExoS antibody (capture Ab); (ii) incubating the 96-well plate with culture supernatant of PAK*exoST*/pHW0029, where pHW0029 encodes an ExoS-FLAG fusion that can be secreted effectively through the type III secretion apparatus (Fig. 4a–c); (iii) binding of the captured ExoS-FLAG with mouse anti-FLAG M2 mAb; (iv) detecting with goat anti-mouse IgG–HRP conjugate; and (v) developing the colorimetric reaction by the addition of substrate OPD (Fig. 4d). Specificity of the sandwich ELISA assay was confirmed by comparison of the assay results with those of the Western blot using anti-FLAG M2 mAb (Fig. 4e–f). To

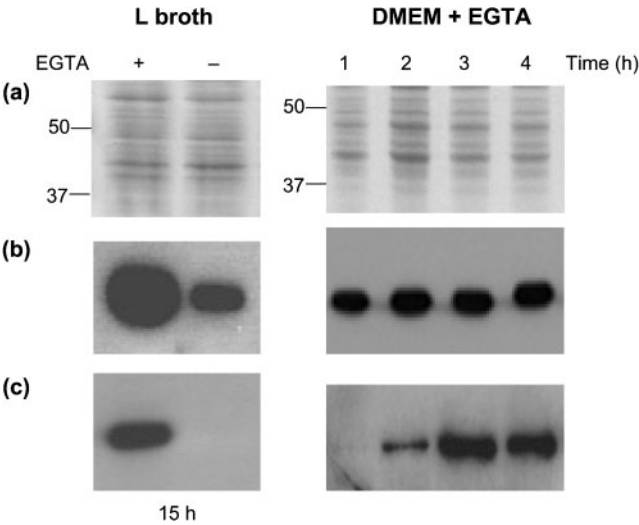


Fig. 2. Expression and secretion of ExoS under type III-inducing conditions. *P. aeruginosa* strain PAKexoT grown for 15 h in L broth with or without 5 mM EGTA was subjected to ExoS analysis (left column). Bacterial cells grown for 15 h in L broth without EGTA were washed in PBS and resuspended in type III-inducing medium (DMEM plus 1% FCS and 5 mM EGTA) with 10-fold dilution. After 1, 2, 3 and 4 h incubation in a CO₂ incubator at 37 °C, the cultures were subjected to ExoS analysis (right column). Bacterial-cell lysates were separated by SDS-PAGE (12% gel) and (a) stained with Coomassie blue or (b) blotted with an anti-ExoS antibody. (c) Secreted ExoS was precipitated with 15% TCA, separated by SDS-PAGE (12% gel) and detected by Western blot using an anti-ExoS antibody.

test the sensitivity of the ELISA assay, ExoS-FLAG protein was purified from the culture supernatant of PAKexoST/pHW0029 using anti-FLAG antibody and protein G-agarose

(see Methods). Based on the analysis of a known amount of purified ExoS-FLAG protein, this ELISA system can detect ExoS-FLAG in culture supernatant from 120 ng ml⁻¹ to 5 µg ml⁻¹ in a linear fashion and the assay system is highly reproducible (Fig. 4g).

By using the newly developed ExoS-FLAG detection system, the requirement of EGTA and L broth or FCS for type III secretion was further investigated. As shown in Fig. 5, secretion of ExoS-FLAG by PAKexoST/pHW0029 was observed only when EGTA and L broth or EGTA and serum were present together, whilst individual components of EGTA or L broth or serum alone were not sufficient, confirming the presence of serum and L broth factors that trigger type III secretion. ExoS-FLAG secretion was seen only in PAK/pHW0029 and PAKexoST/pHW0029 strains, but not in PAKexoA/pHW0029, a strain defective in type III secretion gene activation (Fig. 5), demonstrating a TTSS-specific phenomenon. Here, we designate the serum and L broth factors as type III secretion factors (TSFs).

TSFs in serum and L broth are protein molecules

TSFs in serum and L broth were further characterized by quantitative analysis of the secreted ExoS-FLAG by using the sandwich ELISA system. First, heat stability of the TSF was tested. Boiling did not inactivate the TSF activity of FCS or L broth; instead, the TSF activities increased slightly, indicating that TSF is a heat-stable molecule, possibly a low-molecular-mass compound. Next, the molecular mass range of the TSF was estimated. FCS and L broth were subjected to filtration through a 10 kDa molecular mass cut-off Amicon filter membrane. To our surprise, the TSF activity was found in the high-molecular-mass fraction (>10 kDa). Upon further separation of the high-molecular-mass components by Amicon filters with molecular mass cut-offs of 50 and

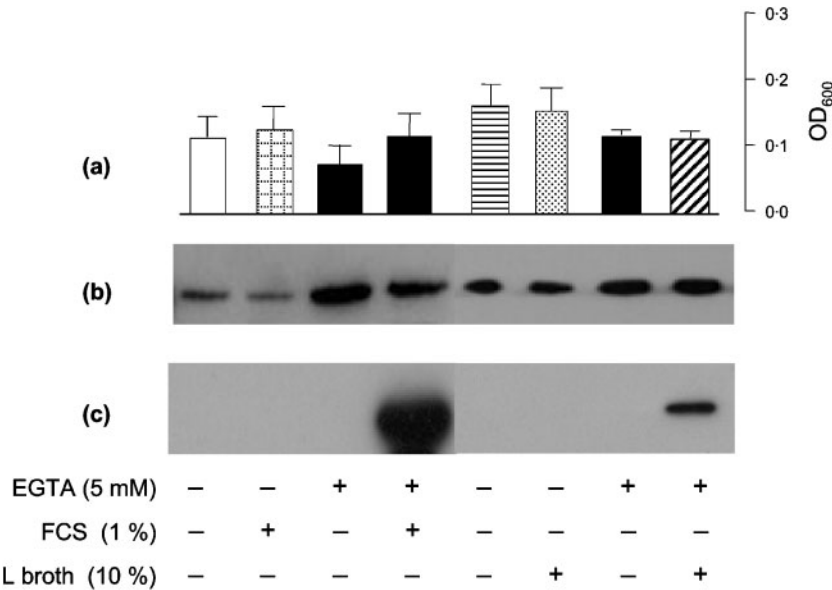


Fig. 3. Secretion of ExoS requires both EGTA and factors from serum or L broth. Strain PAKexoST/pHW0029 was grown in DMEM with indicated supplements for 3 h. Cell density was measured by OD₆₀₀ (a) and cell lysates were separated by SDS-PAGE (12% gel) and blotted with an anti-ExoS antibody to monitor intracellular levels of ExoS (b). Culture supernatants were precipitated with TCA, separated by SDS-PAGE (12% gel) and subjected to Western blot using an anti-ExoS antibody to monitor the secreted form of ExoS (c).

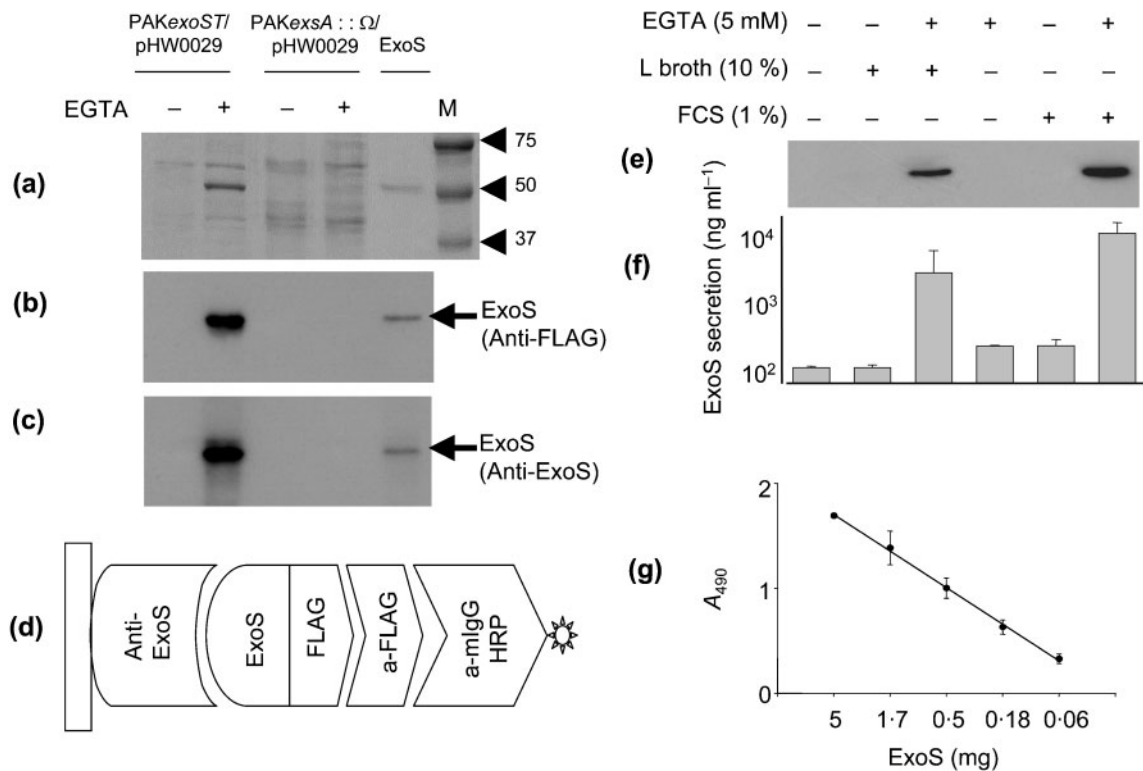


Fig. 4. Evaluation of the sandwich ELISA assay system. PAKexoST and PAKexoA harbouring an *exoS*–*Flag* fusion construct (pHW0029) were grown in L broth in the presence (+) or absence (–) of 5 mM EGTA for 3 h and culture supernatants were precipitated with 15 % TCA. Precipitated proteins, as well as purified ExoS–FLAG protein (0.5 µg), were subjected to SDS-PAGE (12 % gel) and stained with Coomassie blue (a) or blotted with either a mAb against the FLAG tag (b) or a polyclonal antibody against ExoS (c). A sandwich ELISA assay was developed for the detection of ExoS–FLAG in culture supernatants. Anti-ExoS and anti-FLAG were used as capture and detection antibodies, respectively (d). Culture supernatants of PAKexoST/pHW0029 grown in DMEM with indicated supplements for 3 h have been subjected to Western blot using the anti-ExoS antibody (e) or sandwich ELISA (f) to monitor secreted ExoS–FLAG protein. A standard curve correlating the A₄₉₀ values and the quantity of the ExoS–FLAG protein is also shown (g).

100 kDa, the TSF activity of serum was mainly associated with > 50 kDa fractions, whereas that of L broth was associated with 10–50 kDa fractions (Fig. 6a). These

observations apparently contradict the heat-stability data, posing the possibility of small-molecular-mass compounds embedded within the high-molecular-mass molecules. To

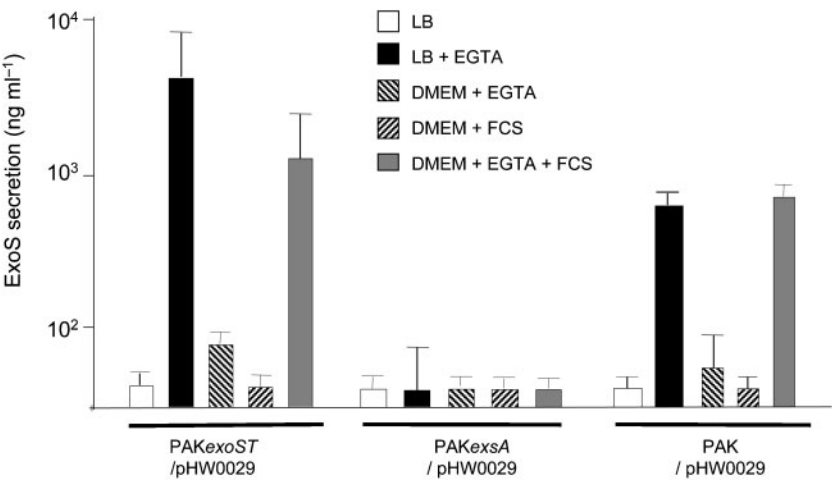


Fig. 5. Effect of FCS and L broth on the expression and secretion of TTSS. Effects of EGTA (5 mM) and FCS (1 %) on the secretion of ExoS–FLAG by strains PAK, PAKexoA and PAKexoSexoT harbouring pHW0029 were evaluated by sandwich ELISA of the culture supernatants. Overnight cultures in L broth were washed and diluted 20-fold in DMEM or L broth with indicated supplements and grown in a CO₂ incubator for 3 h at 37 °C.

test this possibility, serum and L broth were fractionated after boiling for 5 min. Again, the TSF activity remained associated with the original size fractions. We then tested the sensitivity of the TSF to protease treatment. Fractionated FCS and L broth were subjected to protease treatment, then supplemented into DMEM + 5 mM EGTA and used for the ExoS-FLAG secretion assay. As shown in Fig. 6(b), protease treatment completely abolished TSF activity in serum as well as in L broth, suggesting that the TSF is protein in nature.

Albumin in serum is the functional TSF molecule

FCS was further subjected to size fractionation by using an HPLC HR 5/5 column and each fraction was assayed for TSF activity. As shown in Fig. 6(c), the TSF activity of FCS was associated with a single peak in the HPLC fraction, which had a 65 kDa major protein band on a Coomassie blue-stained SDS-PAGE gel. Both N-terminal amino acid sequencing and matrix-assisted laser desorption/ionization-time of flight mass-spectrometry analysis following trypsin digestion determined the protein to be BSA. Commercially available BSA was then tested for TSF activity. Indeed, pure BSA had

all of the observed features of the serum TSF: high TSF activity, heat stability and protease sensitivity. Dose-dependent TSF activities of FCS and BSA were further verified. As seen from Western blots in Fig. 7, the intracellular levels of type III effector molecule ExoS were similar within the initial 3 h culture in DMEM containing 5 mM EGTA at 37 °C (Fig. 7a). However, the secreted form of ExoS was only seen in the presence of FCS or BSA in a concentration-dependent manner (Fig. 7b–c), further confirming the TSF function of albumin in serum. Human serum albumin (HSA) also showed a high TSF activity (Fig. 8a).

To see whether there is any other component besides BSA that exhibits TSF activity, BSA was depleted from FCS by absorbing the BSA through a Blue-Gel affinity column or by using an anti-BSA antibody linked to agarose beads. The BSA-depleted FCS almost completely lost TSF activity, demonstrating that BSA is the major component in FCS that functions as a TSF (Fig. 8b). In a control experiment, incubation of the FCS with anti-HSA antibody-agarose conjugate did not affect the TSF activity of the FCS (Fig. 8b), as anti-HSA antibody does not cross-react with BSA.

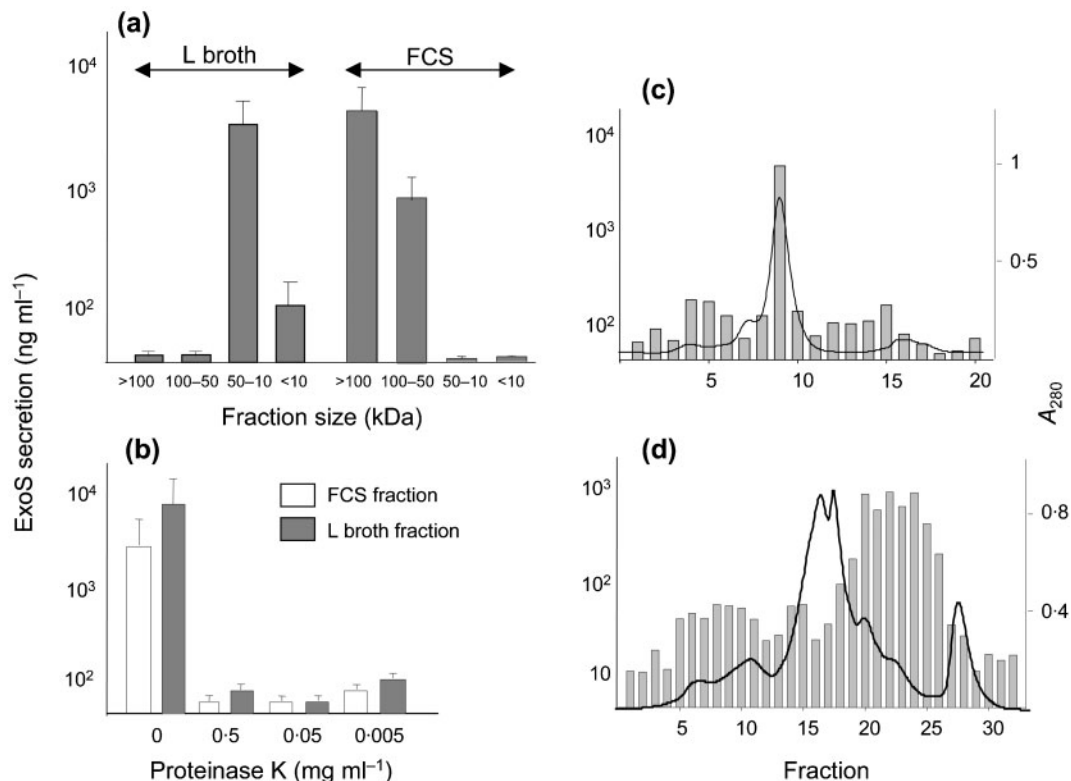


Fig. 6. Identification of the TSFs from serum and L broth. (a) FCS and L broth were size fractionated with Centricon membrane filters (Amicon) with various molecular mass cut-offs and each fraction was tested for TSF activity by using PAK(pHW0029) cultured in DMEM + 5 mM EGTA by sandwich ELISA. (b) The 10–50 kDa fraction of L broth and > 100 kDa fraction of FCS were treated with the indicated concentrations of proteinase K and tested for TSF activity. FCS (c) and L broth (d) were further size fractionated by HPLC and each fraction was tested for TSF activity by the sandwich ELISA. Dashed lines show A₂₈₀ values of elution fractions and bars indicate TSF activity of each fraction.

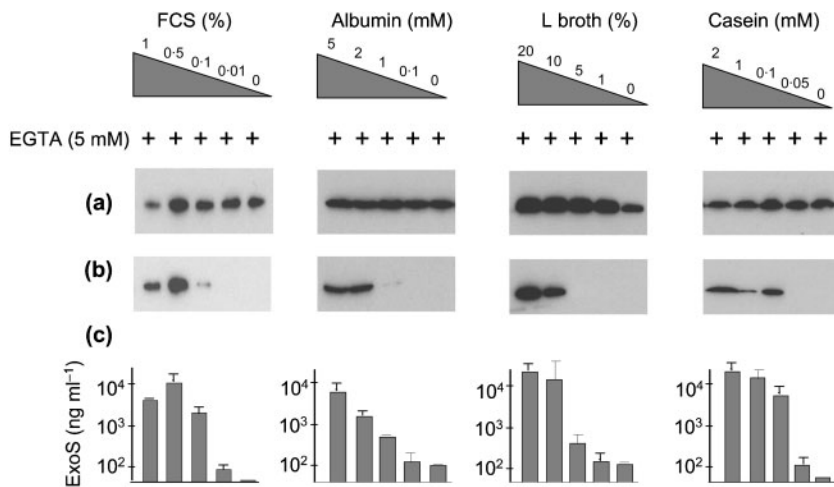


Fig. 7. Dose-dependent activation of type III secretion by FCS, albumin, L broth and casein. Strain PAKexoST(pHW0029) was grown in DMEM containing 5 mM EGTA and the indicated amount of TSF for 3 h and ExoS-FLAG in total bacterial-cell lysates (a) or culture supernatants (b) was detected by Western blot using an anti-FLAG mAb. Secreted ExoS-FLAG in the culture supernatants was also quantified by the sandwich ELISA (c).

Caseins in L broth function as TSFs

Size fractionation of L broth by HPLC showed multiple fractions with TSF activity (Fig. 6d). As the protein

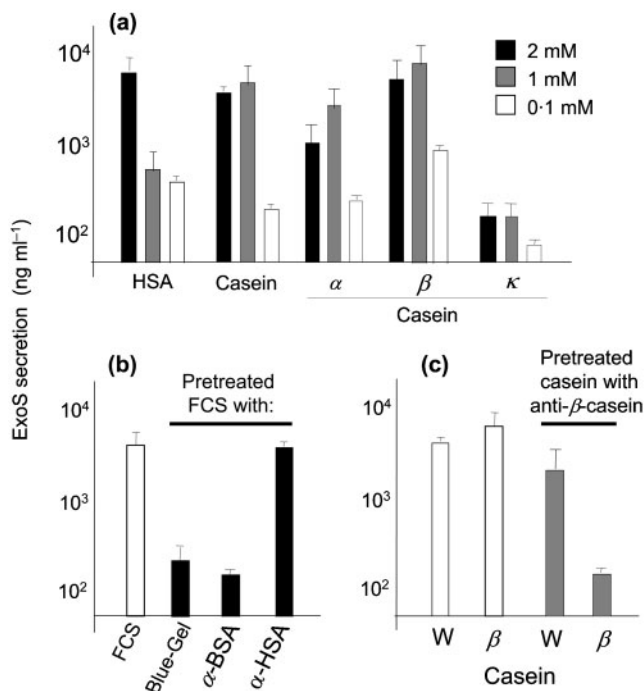


Fig. 8. Characterization of TSF activities of albumin and caseins. (a) TSF activities of HSA and subunits of casein. The indicated amount of HSA and casein subunits were supplemented into DMEM+5 mM EGTA and used to grow PAK(pHW0029) for 3 h at 37 °C in a CO₂ incubator. Culture supernatants were assayed for secreted ExoS-FLAG by sandwich ELISA. (b) FCS was adsorbed with Blue-Gel (binds BSA), anti-BSA or anti-HSA linked to agarose beads and subjected to the TSF-activity test as described above. (c) Whole casein (W) and β -casein (β) were pretreated with anti- β -casein conjugated to agarose beads and subjected to the TSF-activity assay as described above.

components in L broth come from yeast extract and tryptone, we traced the TSF activities of these two components. Interestingly, both yeast extract and tryptone showed TSF activities, with tryptone being more active (data not shown). As tryptone is a pancreatic-digest product of caseins, the result indicated that TSF activity is associated with peptide fragments derived from caseins. Confirming this prediction, casein protein obtained from a commercial source (Sigma) had not only high TSF activity, but also heat-stable and protease-sensitive characters. Dose-dependent TSF activities of L broth and caseins were also verified. As seen from Western blots in Fig. 7, ExoS secretion was only seen in the presence of L broth or casein in a concentration-dependent manner, further confirming the TSF function of the caseins.

The casein complexes of all species contain two types of proteins: the α - and β -caseins, which bind calcium and aggregate, and κ -casein, which does not bind calcium, but stabilizes the α - and β -caseins to yield the micelle or colloid (Swaigood, 1993). Pure individual casein protein components, α -, β - and κ -caseins, were obtained from a commercial source (Sigma) and tested for their TSF activities. As shown in Fig. 8(a), both α - and β -caseins showed high TSF activity, but κ -casein lacked TSF activity, suggesting the possible involvement of Ca²⁺-binding ability in TSF activity. Depletion of the β -casein from whole casein by using an anti- β -casein antibody linked to agarose beads did not eliminate the TSF activity, presumably due to the presence of α -casein. However, adsorption of the β -casein through anti- β -casein antibody-agarose abolished the TSF activity (Fig. 8c), demonstrating that individual casein proteins function as TSFs.

Low-affinity, high-capacity Ca²⁺-binding proteins display TSF activity

There is no apparent amino acid sequence similarity or common structural motif between albumin and casein proteins; thus, the TSF function may involve certain physical properties. One common feature of albumin and casein is

their low-affinity, high-capacity binding of calcium (Aguanno & Ladenson, 1982; Swaisgood, 1993; Vorum *et al.*, 1995; Farrell *et al.*, 2002). To test whether the Ca^{2+} -binding property is associated with the TSF activity, we surveyed a number of well-studied Ca^{2+} -binding proteins, including high- and low-affinity Ca^{2+} -binding proteins. Calcium-binding proteins localized within the cytoplasm (CaBP, CaM, troponin and S100), membrane-anchored (calreticulin and annexin V) and secreted (albumin, casein and α -lactalbumin) have been tested. As shown in Fig. 9, albumin, casein, α -lactalbumin and calreticulin, representing low-affinity Ca^{2+} -binding proteins, showed high TSF activities, whereas annexin V, CaBP, CaM and S100, representing high-affinity Ca^{2+} -binding proteins, exhibited no TSF activity, with the exception of troponin. Troponin is composed of three subunits, C, I and T, of which the C subunit is known to have a high-affinity Ca^{2+} -binding ability (Farah & Reinach, 1995; Borovikov, 1999). Tests of individual subunits of the troponin demonstrated that the TSF activity is not associated with the C subunit; rather, it is associated with the T subunit (Fig. 9). Recently, the troponin T from avian flight muscle has been shown to have a Ca^{2+} -binding activity (Zhang *et al.*, 2004). These data together suggested that low-affinity Ca^{2+} -binding proteins possess TSF activity, although the reason for

requirement of this calcium-binding property for TSF activity is not clear.

DISCUSSION

TTSS of *P. aeruginosa* is highly sensitive to environmental conditions, such as temperature, pH and Ca^{2+} level, as well as metabolic balance (Yahr *et al.*, 1995; Dacheux *et al.*, 2002; Rietsch *et al.*, 2004). In this study, we have shown that type III secretion requires specific signals. When grown overnight in L broth without EGTA, high intracellular levels of the type III effector molecules are accumulated without being secreted. However, addition of TSF and EGTA triggered type III secretion even before the activation of type III gene expression, indicating that active expression of the type III genes, which occurs after 3 h culture (non-shaking in a CO_2 incubator), is not a prerequisite for active secretion and that initial secretion involves pre-existing ExoS molecules. Interestingly, both TSF and EGTA are also required for high-level expression of type III secretion genes by 15 h culture (Fig. 1, left column), thus posing the possibility that type III secretion genes are activated through a secretion-dependent feedback mechanism. Indeed, presence of a functional type III secretion apparatus has been shown to be essential for type III gene expression (Yahr *et al.*, 1996;

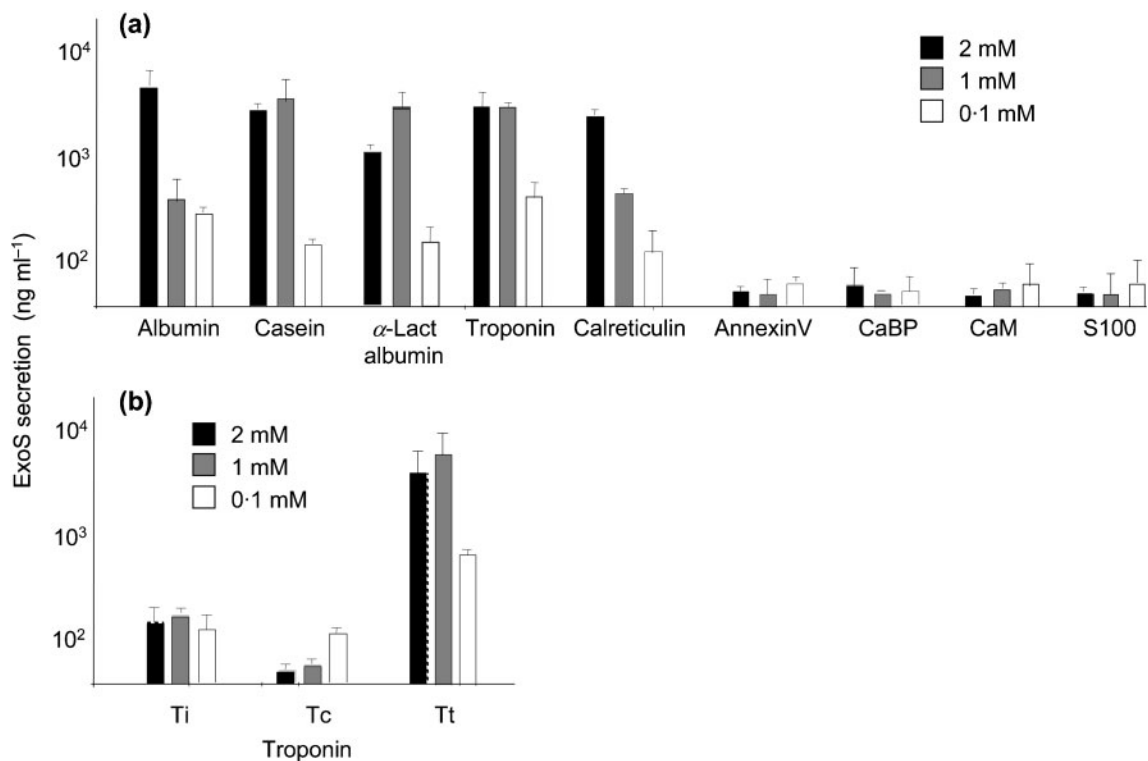


Fig. 9. Survey of Ca^{2+} -binding proteins for TSF activity. (a) Low-affinity, high-capacity Ca^{2+} -binding proteins albumin, casein, α -lactalbumin and calreticulin, as well as high-affinity Ca^{2+} -binding proteins Annexin V, CaBP, CaM, S100 and troponin, were tested for their TSF activities by the sandwich ELISA. (b) Each component of the troponin complex was also tested for the TSF activity.

Vallis *et al.*, 1999). Also, in yersiniae, a feedback regulation has been reported, where the expression of type III genes is lower in type III apparatus-defective mutants. Such feedback control is achieved by secretion of a TTSS-specific suppressor, LcrQ. In the presence of a functional type III secretion apparatus, LcrQ is secreted out of the cell and thus is no longer able to block the expression of TTSS genes, resulting in increased transcription of the TTSS components (Pettersson *et al.*, 1996). Although no LcrQ homologue was found from the genome sequence of *P. aeruginosa*, data from this study, as well as others (Ménard *et al.*, 1994; Yahr *et al.*, 1996; Vallis *et al.*, 1999), suggest the presence of a functional homologue of LcrQ.

To identify factors affecting the TTSS, we have established a sensitive sandwich ELISA system for the detection of secreted ExoS-FLAG and optimized *in vitro* type III-inducing conditions. To test multiple samples at once, bacterial cells were grown in multiwell plates and the culture supernatants were transferred into 96-well microtitre assay plates for direct quantification of secreted ExoS-FLAG. This method of ExoS detection has several advantages over conventional Western blot analysis; first, the assay does not require concentration of the bacterial-culture supernatant; second, it can accurately quantify the amount of secreted ExoS-FLAG protein; and third, hundreds of samples can be assayed at once. With this sensitive and high-throughput assay system, we were able to pursue the mysterious TSF in serum and L broth. This assay system has also proven to be a powerful tool in screening transposon insertional mutant banks for altered type III secretion patterns, which otherwise were impossible to screen for a large number of mutants by using Western blot analysis (J. Kim & S. Jin, unpublished results).

Serum albumin is an abundant, multifunctional protein. It is the major protein component of blood plasma, present at a concentration of around 0.6 mM, but can also be found in bodily tissues and secretions. The protein binds calcium, but its primary role is to transport fatty acids. BSA is a heart-shaped, monomeric protein of 65 kDa and, upon denaturation, it assumes an L-shaped form (Curry *et al.*, 1999). Calcium binding by albumin is a complex process characterized by multiple binding sites whose affinity and binding capacity are variable, depending on parameters such as temperature, pH and ion strength (Fogh-Andersen, 1977; Besarab *et al.*, 1981; Kragh-Hansen & Vorum, 1993). In previous reports, serum has been shown to be essential for the activation of TTSSs of members of the genera *Shigella*, *Salmonella* and *Yersinia* (Ménard *et al.*, 1994; Zierler & Galán, 1995; Lee *et al.*, 2001) and BSA was shown to trigger type III secretion in yersiniae (Lee *et al.*, 2001). Based on the similarities among the TTSSs of these organisms and *P. aeruginosa*, it is likely that the albumin in serum is required for the activation of TTSSs in these bacteria. Also, interestingly, two previous reports have demonstrated a role of BSA and caseins in facilitating the secretion of ATP-utilizing enzymes by mucoid *P. aeruginosa*, as well as *Mycobacterium bovis*, to the surrounding medium (Zaborina

et al., 1999a, b). Relevance of this observation to the activation of type III secretion is not clear.

In eukaryotic cells, cytoplasmic calcium concentrations in a resting state are low (10^{-8} – 10^{-7} M), whilst extracellular concentrations are high (10^{-3} M). Accordingly, intracellular Ca^{2+} -binding proteins have high binding strengths ($\log K=7$), whilst extracellular Ca^{2+} -binding proteins have low binding strengths ($\log K=3$ – 4) (Vogel, 2002). Structurally, a major family of calcium-binding proteins is the 'EF-hand' superfamily, so called because they all contain the 'EF-hand' helix-loop-helix Ca^{2+} -binding motif. Classical EF-hand proteins include calmodulin, parvalbumin and troponin C, whilst non-classical EF-hand proteins include the S100 protein family and calbindins (Persechini *et al.*, 1989; Berchtold, 1993). However, no common structural motifs have been identified among low-affinity Ca^{2+} -binding proteins. As low-affinity Ca^{2+} -binding proteins displayed TSF activity, regardless of their amino acid sequences, this implies the possible involvement of Ca^{2+} -binding activity in regulation of type III secretion. This also explains the observation that yeast extract also contains TSF molecules, as all living organisms encode low-affinity Ca^{2+} -binding proteins. Efforts are under way to identify the minimal functional peptide fragments with TSF activity, with the hope to understand the features essential for TSF function, especially the requirement for a low-affinity Ca^{2+} -binding ability. The observation that high-affinity Ca^{2+} -binding proteins do not have TSF activity suggests that intracellular *P. aeruginosa* is unlikely to secrete type III effectors, which might increase the intracellular life span of the bacteria once inside the host cells.

The process of translocation is contact dependent and occurs without secretion to the surrounding medium during infection of eukaryotic cells (Rosqvist *et al.*, 1994). Translocation has therefore been described as polarized, i.e. secretion takes place only at the zone of contact between the pathogen and the host cell. Based on the fact that many low-affinity Ca^{2+} -binding proteins function as TSFs, the TSF-like molecules are probably abundant in host tissues, even on the host-cell surface; however, a low- Ca^{2+} environment is only found in the microenvironment surrounding host-cell surfaces or host cytosol (Heizmann & Berchtold, 1987; Vogel, 2002). Therefore, through co-evolution with its hosts, *P. aeruginosa* may have chosen abundant host proteins (low-affinity Ca^{2+} -binding proteins) as TSF molecules to perceive the host environment while sensing high- or low- Ca^{2+} environments to distinguish distal vs proximal to the host-cell surface, ensuring type III injection into host cells rather than wasting into the surrounding tissues.

The requirement of TSF and EGTA for TTSS effector secretion indicates the existence of bacterial cell-surface component(s) interacting with the TSF, possibly in a Ca^{2+} concentration-dependent manner. In members of the genus *Yersinia*, three proteins, YopN, LcrG and TyeA, are involved in preventing secretion *in vitro* into Ca^{2+} -containing media and the surrounding culture media during infection of

eukaryotic cells. Strains mutated for any of these genes display de-repressed Yop expression and secretion *in vitro*, i.e. high levels of expression and secretion are seen, irrespective of the Ca^{2+} concentration (Forsberg *et al.*, 1991; Nilles *et al.*, 1997; Day & Plano, 1998; Cheng & Schneewind, 2000; Matson & Nilles, 2001). Differing from the genus *Yersinia*, mutation in the *pcrV* gene of *P. aeruginosa* has been shown to result in constitutive secretion of type III effector molecules (Sawa *et al.*, 1999; McCaw *et al.*, 2002). However, no interactions between PcrV and the identified TSF molecules were observed (J. Kim & S. Jin, unpublished results), suggesting alternative target molecules for the TSF. Efforts are under way to identify the TSF-binding bacterial cell-surface component(s) involved in the control of type III secretion.

ACKNOWLEDGEMENTS

This work is supported by grants from the American Cancer Society and the Cystic Fibrosis Foundation (to S.J.).

REFERENCES

- Aballay, A. & Ausubel, F. M. (2002). *Caenorhabditis elegans* as a host for the study of host–pathogen interactions. *Curr Opin Microbiol* **5**, 97–101.
- Aguanno, J. J. & Ladenson, J. H. (1982). Influence of fatty acids on the binding of calcium to human albumin: correlation of binding and conformation studies and evidence for distinct differences between unsaturated fatty acids and saturated fatty acids. *J Biol Chem* **257**, 8745–8748.
- Ahn, K.-S., Ha, U., Jia, J., Wu, D. & Jin, S. (2004). The *truA* gene of *Pseudomonas aeruginosa* is required for the expression of type III secretory genes. *Microbiology* **150**, 539–547.
- Berchtold, M. W. (1993). Evolution of EF-hand calcium-modulated proteins. V. The genes encoding EF-hand proteins are not clustered in mammalian genomes. *J Mol Evol* **36**, 489–496.
- Besarab, A., DeGuzman, A. & Swanson, J. W. (1981). Effect of albumin and free calcium concentrations on calcium binding *in vitro*. *J Clin Pathol* **34**, 1361–1367.
- Bodey, G. P., Bolivar, R., Fainstein, V. & Jadeja, L. (1983). Infections caused by *Pseudomonas aeruginosa*. *Rev Infect Dis* **5**, 279–313.
- Borovikov, Y. S. (1999). Conformational changes of contractile proteins and their role in muscle contraction. *Int Rev Cytol* **189**, 267–301.
- Cheng, L. W. & Schneewind, O. (2000). *Yersinia enterocolitica* TyeA, an intracellular regulator of the type III machinery, is required for specific targeting of YopE, YopH, YopM, and YopN into the cytosol of eukaryotic cells. *J Bacteriol* **182**, 3183–3190.
- Coburn, J. & Gill, D. M. (1991). ADP-ribosylation of p21^{ras} and related proteins by *Pseudomonas aeruginosa* exoenzyme S. *Infect Immun* **59**, 4259–4262.
- Curry, S., Brick, P. & Franks, N. P. (1999). Fatty acid binding to human serum albumin: new insights from crystallographic studies. *Biochim Biophys Acta* **1441**, 131–140.
- Dacheux, D., Epaulard, O., de Groot, A., Guery, B., Leberre, R., Attree, I., Polack, B. & Toussaint, B. (2002). Activation of the *Pseudomonas aeruginosa* type III secretion system requires an intact pyruvate dehydrogenase *aceAB* operon. *Infect Immun* **70**, 3973–3977.
- D'Argenio, D. A., Gallagher, L. A., Berg, C. A. & Manoil, C. (2001). *Drosophila* as a model host for *Pseudomonas aeruginosa* infection. *J Bacteriol* **183**, 1466–1471.
- Davis, B. D. & Mingioli, E. S. (1950). Mutants of *Escherichia coli* requiring methionine or vitamin B₁₂. *J Bacteriol* **60**, 17–28.
- Day, J. B. & Plano, G. V. (1998). A complex composed of SycN and YscB functions as a specific chaperone for YopN in *Yersinia pestis*. *Mol Microbiol* **30**, 777–788.
- Deretic, V., Schurr, M. J. & Yu, H. (1995). *Pseudomonas aeruginosa*, mucoidy and the chronic infection phenotype in cystic fibrosis. *Trends Microbiol* **3**, 351–356.
- Farah, C. S. & Reinach, F. C. (1995). The troponin complex and regulation of muscle contraction. *FASEB J* **9**, 755–767.
- Farrell, H. M., Jr, Kumosinski, T. F., Malin, E. L. & Brown, E. M. (2002). The caseins of milk as calcium-binding proteins. *Methods Mol Biol* **172**, 97–140.
- Finck-Barbancon, V., Goranson, J., Zhu, L., Sawa, T., Wiener-Kronish, J. P., Fleiszig, S. M., Wu, C., Mende-Mueller, L. & Frank, D. W. (1997). ExoU expression by *Pseudomonas aeruginosa* correlates with acute cytotoxicity and epithelial injury. *Mol Microbiol* **25**, 547–557.
- Fogh-Andersen, N. (1977). Albumin/calcium association at different pH, as determined by potentiometry. *Clin Chem* **23**, 2122–2126.
- Forsberg, Å., Viitanen, A. M., Skurnik, M. & Wolf-Watz, H. (1991). The surface-located YopN protein is involved in calcium signal transduction in *Yersinia pseudotuberculosis*. *Mol Microbiol* **5**, 977–986.
- Frank, D. W. (1997). The exoenzyme S regulon of *Pseudomonas aeruginosa*. *Mol Microbiol* **26**, 621–629.
- Frank, D. W., Nair, G. & Schweizer, H. P. (1994). Construction and characterization of chromosomal insertional mutations of the *Pseudomonas aeruginosa* exoenzyme S *trans*-regulatory locus. *Infect Immun* **62**, 554–563.
- Frithz-Lindsten, E., Du, Y., Rosqvist, R. & Forsberg, Å. (1997). Intracellular targeting of exoenzyme S of *Pseudomonas aeruginosa* via type III-dependent translocation induces phagocytosis resistance, cytotoxicity and disruption of actin microfilaments. *Mol Microbiol* **25**, 1125–1139.
- Frithz-Lindsten, E., Holmström, A., Jacobsson, L., Soltani, M., Olsson, J., Rosqvist, R. & Forsberg, Å. (1998). Functional conservation of the effector protein translocators PopB/YopB and PopD/YopD of *Pseudomonas aeruginosa* and *Yersinia pseudotuberculosis*. *Mol Microbiol* **29**, 1155–1165.
- Ganesan, A. K., Frank, D. W., Misra, R. P., Schmidt, G. & Barbieri, J. T. (1998). *Pseudomonas aeruginosa* exoenzyme S ADP-ribosylates Ras at multiple sites. *J Biol Chem* **273**, 7332–7337.
- Ha, U. & Jin, S. (2001). Growth phase-dependent invasion of *Pseudomonas aeruginosa* and its survival within HeLa cells. *Infect Immun* **69**, 4398–4406.
- Ha, U.-H., Kim, J., Badrane, H., Jia, J., Baker, H. V., Wu, D. & Jin, S. (2004). An *in vivo* inducible gene of *Pseudomonas aeruginosa* encodes an anti-ExsA to suppress the type III secretion system. *Mol Microbiol* **54**, 307–320.
- Hauser, A. R., Kang, P. J. & Engel, J. N. (1998). PepA, a secreted protein of *Pseudomonas aeruginosa*, is necessary for cytotoxicity and virulence. *Mol Microbiol* **27**, 807–818.
- Heizmann, C. W. & Berchtold, M. W. (1987). Expression of parvalbumin and other Ca^{2+} -binding proteins in normal and tumor cells: a topical review. *Cell Calcium* **8**, 1–41.
- Hogardt, M., Roeder, M., Schreff, A. M., Eberl, L. & Heesemann, J. (2004). Expression of *Pseudomonas aeruginosa* *exoS* is controlled by quorum sensing and RpoS. *Microbiology* **150**, 843–851.

- Holder, I. A. (1993). *Pseudomonas aeruginosa* virulence associated factors and their role in burn wound infections. In *Pseudomonas aeruginosa, the Opportunist: Pathogenesis and Disease*, pp. 235–245. Edited by R. B. Fick, Jr. Boca Raton, FL: CRC Press.
- Hovey, A. K. & Frank, D. W. (1995). Analyses of the DNA-binding and transcriptional activation properties of ExsA, the transcriptional activator of the *Pseudomonas aeruginosa* exoenzyme S regulon. *J Bacteriol* **177**, 4427–4436.
- Hueck, C. J. (1998). Type III protein secretion systems in bacterial pathogens of animals and plants. *Microbiol Mol Biol Rev* **62**, 379–433.
- Jia, J., Alaoui-El-Azher, M., Chow, M., Chambers, T. C., Baker, H. & Jin, S. (2003). c-Jun NH₂-terminal kinase-mediated signaling is essential for *Pseudomonas aeruginosa* ExoS-induced apoptosis. *Infect Immun* **71**, 3361–3370.
- Kaufman, M. R., Jia, J., Zeng, L., Ha, U., Chow, M. & Jin, S. (2000). *Pseudomonas aeruginosa* mediated apoptosis requires the ADP-ribosylating activity of ExoS. *Microbiology* **146**, 2531–2541.
- Kragh-Hansen, U. & Vorum, H. (1993). Quantitative analyses of the interaction between calcium ions and human serum albumin. *Clin Chem* **39**, 202–208.
- Lee, V. T., Mazmanian, S. K. & Schneewind, O. (2001). A program of *Yersinia enterocolitica* type III secretion reactions is activated by specific signals. *J Bacteriol* **183**, 4970–4978.
- Lory, S. & Strom, M. S. (1997). Structure-function relationship of type-IV prepilin peptidase of *Pseudomonas aeruginosa* – a review. *Gene* **192**, 117–121.
- Matson, J. S. & Nilles, M. L. (2001). LcrG-LcrV interaction is required for control of Yops secretion in *Yersinia pestis*. *J Bacteriol* **183**, 5082–5091.
- McCaw, M. L., Lykken, G. L., Singh, P. K. & Yahr, T. L. (2002). ExsD is a negative regulator of the *Pseudomonas aeruginosa* type III secretion regulon. *Mol Microbiol* **46**, 1123–1133.
- Ménard, R., Sansonetti, P. & Parsot, C. (1994). The secretion of the *Shigella flexneri* Ipa invasins is activated by epithelial cells and controlled by IpaB and IpaD. *EMBO J* **13**, 5293–5302.
- Miller, J. H. (1972). *Experiments in Molecular Genetics*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.
- Muller, S., Feldman, M. F. & Cornelis, G. R. (2001). The type III secretion system of Gram-negative bacteria: a potential therapeutic target? *Expert Opin Ther Targets* **5**, 327–339.
- Nilles, M. L., Williams, A. W., Skrzypek, E. & Straley, S. C. (1997). *Yersinia pestis* LcrV forms a stable complex with LcrG and may have a secretion-related regulatory role in the low-Ca²⁺ response. *J Bacteriol* **179**, 1307–1316.
- Pederson, K. J., Vallis, A. J., Aktories, K., Frank, D. W. & Barbieri, J. T. (1999). The amino-terminal domain of *Pseudomonas aeruginosa* ExoS disrupts actin filaments via small-molecular-weight GTP-binding proteins. *Mol Microbiol* **32**, 393–401.
- Persechini, A., Moncrief, N. D. & Kretsinger, R. H. (1989). The EF-hand family of calcium-modulated proteins. *Trends Neurosci* **12**, 462–467.
- Pettersson, J., Nordfelth, R., Dubinina, E., Bergman, T., Gustafsson, M., Magnusson, K. E. & Wolf-Watz, H. (1996). Modulation of virulence factor expression by pathogen target cell contact. *Science* **273**, 1231–1233.
- Pier, G. B. (2002). CFTR mutations and host susceptibility to *Pseudomonas aeruginosa* lung infection. *Curr Opin Microbiol* **5**, 81–86.
- Rahme, L. G., Stevens, E. J., Wolfort, S. F., Shao, J., Tompkins, R. G. & Ausubel, F. M. (1995). Common virulence factors for bacterial pathogenicity in plants and animals. *Science* **268**, 1899–1902.
- Ramphal, R., Koo, L., Ishimoto, K. S., Totten, P. A., Lara, J. C. & Lory, S. (1991). Adhesion of *Pseudomonas aeruginosa* pilin-deficient mutants to mucin. *Infect Immun* **59**, 1307–1311.
- Rietsch, A., Wolfgang, M. C. & Mekalanos, J. J. (2004). Effect of metabolic imbalance on expression of type III secretion genes in *Pseudomonas aeruginosa*. *Infect Immun* **72**, 1383–1390.
- Rosqvist, R., Magnusson, K.-E. & Wolf-Watz, H. (1994). Target cell contact triggers expression and polarized transfer of *Yersinia* YopE cytotoxin into mammalian cells. *EMBO J* **13**, 964–972.
- Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989). *Molecular Cloning: a Laboratory Manual*, 2nd edn. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.
- Sato, H., Feix, J. B., Hillard, C. J. & Frank, D. W. (2005). Characterization of phospholipase activity of the *Pseudomonas aeruginosa* type III cytotoxin, ExoU. *J Bacteriol* **187**, 1192–1195.
- Sawa, T., Yahr, T. L., Ohara, M., Kurahashi, K., Gropper, M. A., Wiener-Kronish, J. P. & Frank, D. W. (1999). Active and passive immunization with the *Pseudomonas* V antigen protects against type III intoxication and lung injury. *Nat Med* **5**, 392–398.
- Schweizer, H. P. (1991). *Escherichia-Pseudomonas* shuttle vectors derived from pUC18/19. *Gene* **97**, 109–121.
- Swaisgood, H. E. (1993). Review and update of casein chemistry. *J Dairy Sci* **76**, 3054–3061.
- Totten, P. A. & Lory, S. (1990). Characterization of the type a flagellin gene from *Pseudomonas aeruginosa* PAK. *J Bacteriol* **172**, 7188–7199.
- Vallis, A. J., Yahr, T. L., Barbieri, J. T. & Frank, D. W. (1999). Regulation of ExoS production and secretion by *Pseudomonas aeruginosa* in response to tissue culture conditions. *Infect Immun* **67**, 914–920.
- Vasil, M. L. & Ochsner, U. A. (1999). The response of *Pseudomonas aeruginosa* to iron: genetics, biochemistry and virulence. *Mol Microbiol* **34**, 399–413.
- Vogel, H. J. (2002). *Calcium-binding Protein Protocols* (Methods in Molecular Biology no. 173), pp. 172–173. Totowa, NJ: Humana Press.
- Vorum, H., Fisker, K., Otagiri, M., Pedersen, A. O. & Kragh-Hansen, U. (1995). Calcium ion binding to clinically relevant chemical modifications of human serum albumin. *Clin Chem* **41**, 1654–1661.
- Wolfgang, M. C., Lee, V. T., Gilmore, M. E. & Lory, S. (2003). Coordinate regulation of bacterial virulence genes by a novel adenylate cyclase-dependent signaling pathway. *Dev Cell* **4**, 253–263.
- Yahr, T. L. & Frank, D. W. (1994). Transcriptional organization of the *trans*-regulatory locus which controls exoenzyme S synthesis in *Pseudomonas aeruginosa*. *J Bacteriol* **176**, 3832–3838.
- Yahr, T. L., Hovey, A. K., Kulich, S. M. & Frank, D. W. (1995). Transcriptional analysis of the *Pseudomonas aeruginosa* exoenzyme S structural gene. *J Bacteriol* **177**, 1169–1178.
- Yahr, T. L., Goranson, J. & Frank, D. W. (1996). Exoenzyme S of *Pseudomonas aeruginosa* is secreted by a type III pathway. *Mol Microbiol* **22**, 991–1003.
- Yahr, T. L., Vallis, A. J., Hancock, M. K., Barbieri, J. T. & Frank, D. W. (1998). ExoY, an adenylate cyclase secreted by the *Pseudomonas aeruginosa* type III system. *Proc Natl Acad Sci U S A* **95**, 13899–13904.
- Zaborina, O., Li, X., Cheng, G., Kapatal, V. & Chakrabarty, A. M. (1999a). Secretion of ATP-utilizing enzymes, nucleoside diphosphate kinase and ATPase, by *Mycobacterium bovis* BCG: sequestration of ATP from macrophage P2Z receptors? *Mol Microbiol* **31**, 1333–1343.

Zaborina, O., Misra, N., Kostal, J., Kamath, S., Kapatral, V., El-Idrissi, M. E.-A., Prabhakar, B. S. & Chakrabarty, A. M. (1999b). P2Z-independent and P2Z receptor-mediated macrophage killing by *Pseudomonas aeruginosa* isolated from cystic fibrosis patients. *Infect Immun* **67**, 5231–5242.

Zhang, Z., Jin, J.-P. & Root, D. D. (2004). Binding of calcium ions to an avian flight muscle troponin T. *Biochemistry* **43**, 2645–2655.

Zierler, M. K. & Galán, J. E. (1995). Contact with cultured epithelial cells stimulates secretion of *Salmonella typhimurium* invasion protein InvJ. *Infect Immun* **63**, 4024–4028.